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FULL RESEARCH PAPER

Parasitism of *Trichoderma* on *Meloidogyne javanica* and role of the gelatinous matrix

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Abstract *Trichoderma* (*T. asperellum*-203, 44 and GH11; *T. atroviride*-IMI 206040 and *T. harzianum*-248) parasitism on *Meloidogyne javanica* life stages was examined in vitro. Conidium attachment and parasitism differed beween the fungi. Egg masses, their derived eggs and second-stage juveniles (J2) were parasitized by *Trichoderma asperellum*-203, 44, and *T. atroviride* following conidium attachment. *Trichoderma asperellum*-GH11 attached to the nematodes but exhibited reduced penetration, whereas growth of *T. harzianum*-248 attached to egg masses was inhibited. Only a few conidia of the different fungi were attached to eggs and J2s without gelatinous matrix; the eggs were penetrated and parasitized by few hyphae, while J2s were rarely parasitized by the fungi. The gelatinous matrix specifically induced J2 immobilization by *T. asperellum*-203, 44 and *T. atroviride* metabolites that immobilized the J2s. A constitutive-GFP-expressing *T. asperellum*-203 construct was used to visualize fungal penetration of the nematodes. Scanning electron microscopy revealed the formation of coiling and appressorium-like structures upon attachment and parasitism by *T. asperellum*-203 and *T. atroviride*. Gelatinous matrix agglutinated *T. asperellum*-203 and *T. atroviride* conidia, a process that was Ca^{2+}-dependent. Conidium agglutination was inhibited by carbohydrates, including fucose, as was conidium attachment to the nematodes. All but *T. harzianum* could grow on the gelatinous matrix, which enhanced conidium germination. A biomimetic system based on gelatinous-matrix-coated nylon fibers demonstrated the role of the matrix in parasitism: *T. asperellum*-203 and *T. atroviride* conidia attached specifically to the gelatinous-matrix-coated fibers and parasitic growth patterns, such as coiling, branching and appressoria-like structures, were induced in both fungi, similarly to those observed during nematode parasitism. All *Trichoderma* isolates exhibited nematode biocontrol activity in pot experiments with tomato plants. Parasitic interactions were demonstrated *in planta*: females and egg masses dissected from tomato roots grown in *T. asperellum*-203-treated soil were examined and found to be parasitized by the fungus. This study demonstrates biocontrol activities of

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Trichoderma isolates and their parasitic capabilities on *M. javanica*, elucidating the importance of the gelatinous matrix in the fungal parasitism.

Keywords Attachment · Biological control · Carbohydrates · Recognition

Abbreviations

gm	Gelatinous matrix
J2	Second-stage juveniles
RKN	Root-knot nematode

Introduction

Plant-parasitic nematodes cause great economic losses to agricultural crops worldwide. Root-knot nematodes (RKNs, *Meloidogyne* spp.) are sedentary, polyphagous root endoparasites. Species such as *M. javanica* and *M. incognita* are among the major limiting factors in the production of field and plantation crops. The RKN second-stage juveniles (J2s), which penetrate and develop in the roots, induce a cascade of changes in the host plant that lead to the formation of giant cells and galls. About 1 month after J2 penetration, eggs are laid, embedded within masses in a gelatinous matrix (gm) secreted by the female. Soil pathogens are difficult to control, and the RKNs pose particular difficulties because of their wide host range, short generation times, high reproductive rates and endoparasitic nature (Trudgill and Blok 2001; Manzanilla-Lopez et al. 2004).

Trichoderma species are free-living fungi that are common in soil and root ecosystems. They are opportunistic, avirulent plant symbionts, as well as parasites of other fungi. Some strains establish robust and long-lasting colonizations of root surfaces and penetrate into the epidermis and a few cells below this level. Root colonization by *Trichoderma* spp. frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and uptake and use of nutrients (Harman et al. 2004). Various mechanisms have been suggested for the biocontrol activity of *Trichoderma* against phytopathogenic fungi: antibiosis, competition, enzymatic hydrolysis, parasitism and systemic induced resistance (Chet et al. 1997; Harman et al. 2004).

Several attempts have been made to use *Trichoderma* as a biocontrol agent against plant-parasitic nematodes (Windham et al. 1989, Reddy et al. 1996, Rao et al. 1998). Direct interactions between *T. harzianum* and the potato cyst nematode *Globodera rostochiensis* have been demonstrated in vitro by Saifullah and Thomas (1996). Biocontrol activities of *T. asperellum*-203 and *T. atroviride* IMI 206040 (both fungi were previously defined as strains of *T. harzianum*) have been reported against *M. javanica* in soil (Sharon et al. 2001). Other *Trichoderma* species and isolates have also exhibited significant biocontrol activity against *M. javanica* in growth-chamber experiments (Spiegel et al. 2006).

The ability of *T. asperellum*-203 and *T. atroviride* to parasitize nematode eggs and J2s has been observed (Sharon et al. 2001); therefore, especial emphasis was placed in this study on these two species. Mechanisms involved in the attachment and parasitism processes were investigated, with special attention to the role of the gm in direct nematode-fungus interactions.

Materials and methods

Nematodes

Monoxenic cultures of the nematode were grown aseptically on excised tomato roots in Petri dishes on Gamborg-B5 medium (Duchefa, Haarlem, the Netherlands) which contained sucrose (20 g l^{-1}) and 0.75% (w/v) Gelrite (an agar substitute; Duchefa), and kept in an incubator at 25 ± 1°C. These cultures were used to obtain nematode egg masses and gm. Egg masses were crushed to obtain gm-originated eggs. Separated eggs (designated herein as gm-free eggs) were extracted from nematode-infected roots by shaking with 0.5% sodium hypochlorite (NaOCl) solution for 1 min. Eggs were collected on a 30 µm sieve and washed thoroughly with water. Pre-infective J2s were hatched either from gm-free eggs (designated herein as gm-free J2), or directly from egg masses (designated herein as gm-J2), on a 30 µm nylon sieve, in water.

Trichoderma

Fungal cultures were grown on potato dextrose agar (PDA) (Difco™, Becton Dickinson, Sparks, MD, USA) in 9 cm diam Petri plates. Conidia were

collected from the plates in water. Preparations, made on a mixture of peat and wheat bran (1:1), containing 10^8 CFU g^{-1}, were obtained as described in Sivan et al. (1984).

Species and isolates of *Trichoderma*: I. *T. atroviride* IMI 206040 was provided by Prof. A. Herrera-Estrella, Mexico. II. *T. asperellum*-203 and a green fluorescent protein (GFP) construct of this isolate (*gfp::pki1* under control of the constitutive pyruvate kinase promoter). These two *Trichoderma* species had been previously reported in the literature as strains of *T. harzianum* and were reidentified (Kullnig et al. 2001; Rocha-Ramirez et al. 2002). These species were used before in nematode biocontrol studies (Sharon et al. 2001). The following three cultures have not been reported previously and were newly identified: III. *T. asperellum*-44. IV. *T. asperellum*-GH11, and V. *T. harzianum*-248.

Identification of *Trichoderma* species: *Trichoderma* cultures of III, IV and V were identified by microscopy and then by DNA sequencing. Approximately 600 base pairs of the translation-elongation factor 1-alpha (TEF-1alpha) gene were sequenced. This region includes one large intron and two exons. The primers used were: tef1-728 (Carbone and Kohn 1999) and tef1 rev (Samuels et al. 2002). These gave a PCR product of about 600 bp, sequenced in both directions.

All the isolates used in this study are also biocontrol isolates against plant fungal diseases; activities of I and II have been studied and published and fungal biocontrol by III, IV and V was demonstrated (Chet, unpubl.).

In vitro parasitism and attachment bioassays

Attachment and parasitism of the *Trichoderma* species and isolates were bioassayed on various life stages of *M. javanica* in 96-well plates. The plates contained 80 µl of diluted medium [20-fold diluted potato dextrose broth (PDB) (Difco^{TM}); 0.05% w/v KCl; 0.05% w/v $MgSO_4 \cdot 7H_2O$; 1 mM $CaCl_2$], 10 µl of an aqueous suspension of 10^5 fungal conidia ml^{-1}, and about 100 J2s or eggs in 10 µl water, or two egg masses. This diluted medium was designed as a minimal medium to support fungal germination and sparse growth, which enabled microscopic observations and fungal growth in control treatments without nematodes (a 20-fold PDB dilution was selected for suitable growth after testing several other dilutions between 10 and 30-fold). There were five replicates for each treatment. Controls consisted of nematodes without the fungi and/or fungi without the nematodes. Percentages of parasitized nematode eggs and J2s were determined after 48 h, using an inverted microscope. Attachment of fungal conidia to various nematode life stages was observed.

Preparation of gm suspension

Egg masses were dissected from 6 week-old monoxenic cultures of *M. javanica* and suspended in distilled water (25 egg masses ml^{-1}). The matrix was separated from the egg masses by vigorously shaking the suspension for 1 min with a Vortex apparatus and then centrifuging at 1000×*g* for 1 min. The supernatant fraction was separated from the eggs and used immediately or kept at −20°C until use. Total protein concentration in the gm suspension was determined using a protein assay reagent (BioRad Laboratories, Hercules, CA, USA).

Conidia-agglutination assays

Agglutination assays were performed in round-bottom 96-well plates with gm suspension in serial twofold dilutions. Each well contained 50 µl of gm suspension, 50 µl of conidial suspension, and 100 µl PBS pH 7.4 containing 2 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$, or Ca^{2+}- Mg^{2+}- and Mn^{2+}-free PBS, or PBS containing each of the ions separately. Conidial suspensions of *T. asperellum*-203 or *T. atroviride* contained ca. 10^6 conidial ml^{-1} and were adjusted to obtain clear conidial sediment in the control.

Effect of carbohydrates on conidia agglutination by gm and on attachment to nematodes

The effect of carbohydrates on agglutination was assayed by pre-incubating conidia of *T. asperellum*-203 for 30 min with 0.1 M of each one of the following carbohydrates in Ca^{2+}-containing PBS: L-fucose, α-methyl-manoside, glucose, galactose, *N*-acetyl-glucosamine and *N*-acetyl-galactosamine. Conidia were washed by centrifugation and subjected to agglutination assays. The effect of carbohydrates on attachment of conidia of *T. asperellum*-203 to nematodes was evaluated with L-fucose and

α-methyl-manoside, which were incorporated at 0.1 M concentration into a parasitism-like assay system with gm-J2s, in Ca^{2+}-containing PBS. Conidia pre-treated with those carbohydrates were also tested.

Effect of periodate treatment on conidial attachment to nematodes

Nematodes (egg masses and their derived eggs and J2s) and conidia of *T. asperellum*-203 were each incubated in 10 mM sodium periodate ($NaIO_4$) in 50 mM citrate buffer pH 4.6 for 90 min on ice, in the dark. Nematodes and conidia were then thoroughly washed with distilled water and subjected to attachment bioassays.

Nylon fiber biomimetic system

The nylon fiber biomimetic system, originally developed by Inbar and Chet (1992) to mimic fungal-fungal interactions, was modified and used to biomimic nematode-fungus interactions. Nylon 66 fibers (approximate diam, 14 μm; kindly supplied by Nilit, Migdal-Haemek, Israel) were prepared as described by Omero et al. (1999). The fibers were treated with gm suspension containing 100 μg protein ml^{-1} or, as a control, bovine serum albumin (BSA) at the same concentration for 30 min, and then air-dried. Assays were performed in 24-well plates under the conditions described above for parasitism. Each well contained one grid in 500 μl of diluted medium and 50 μl of aqueous conidial suspension containing 10^5 conidia ml^{-1}. The plates were gently agitated for 1 h and then the grids, with or without the attached conidia, were transferred to fresh medium and incubated at 27 ± 1°C for 24 h.

Laser scanning confocal microscopy (LSCM)

In bioassays monitoring GFP-fungal constructs, an Olympus IX 81 inverted laser scanning confocal microscope (Olympus, Japan) equipped with a 488 nm argon-ion laser was used for observation and image acquisition. GFP was excited by 488 nm light and the emission was collected through a BA 515-525 filter. To observe autofluorescence, a BA 660 IF emission filter (red) was used. Confocal optical sections were obtained at 0.5 μm increments, and 3-D images were generated using the Flowview 500 software. Parasitism bioassays for LSCM were performed in glass-bottom 35 mm microwell dishes (MatTek Corporation, Ashland, MA, USA).

Scanning electron microscopy (SEM)

Trichoderma parasitism on nematodes and fungal behaviour in the nylon biomimetic system were observed by SEM [JEOL JSM 5410LV (low vacuum), Tokyo, Japan]. Samples were vapour-fixed with 2.5% glutaraldehyde and air-dried for 24 h, after which they were coated with gold palladium in a model E-5150 sputter coater (Polaron Equipment, Watford, UK) and examined by SEM. Parasitism bioassays for SEM observations were conducted in 12-well plates under the conditions described above. Nematodes were placed on 10 μm sterile nylon sieves or on cellophane pieces, which were then transferred for sample preparation.

Examination of fungal interactions with nematodes *in planta*

Observations were made on sterile tomato seedlings during nematode penetration. Surface-sterilized tomato seeds were germinated in sterile water; the germ roots were then dipped in a *T. asperellum*-203-GFP conidial suspension containing 10^5 conidia ml^{-1} and planted in 10 ml of autoclaved soil placed in 15 ml tubes, in six replicates. After 3 days, the soil was inoculated with 50 sterile J2s, which had previously hatched in monoxenic cultures. Controls contained seedlings with fungus or nematodes alone. Roots were examined with a fluorescent confocal microscope 3 days after nematode inoculation and numbers of galls per seedlings were recorded. Nematodes on roots from growth-chamber experiments were examined as described below.

Nematode biocontrol by *Trichoderma* in soil experiments in growth chambers were performed in 1.5 l pots with peat-bran preparations of the various *Trichoderma* species and isolates. Fungal preparations were mixed with nematode-infested soil at a concentration of 1% (w/w) 2 weeks before tomato speed-seedlings cv. 144 (Hishtill Nursery, Ashkelon, Israel) were planted. Non-treated, nematode-infested soil and peat-bran-amended soil served as controls. Each treatment included eight replicates. Six weeks after planting, the plants were uprooted and their

roots examined. Samples of females and egg masses from roots (10 per root) were dissected for microscopic observations. Eggs from each root were than collected after 0.5% hypochlorite treatment and examined. Samples (about 100 eggs in five replicates) from the eggs were incubated in 1 ml water for 2 days and hatched J2s were counted.

Results

Parasitism of *Trichoderma* isolates on different nematode life stages: in vitro bioassays

Egg masses, eggs and J2 from egg masses were exposed to different *Trichoderma* species and isolates. Some of these fungi were parasites: conidia of *T. atroviride* and *T. asperellum* isolates 203 and 44 adhered to the gm around the egg masses (Fig. 1a, g) and prolific fungal growth was observed upon parasitism of the egg masses (Fig. 1g): germinating hyphae penetrated the egg masses and parasitized the eggs and J2s within them. Conidial attachment to gm-originated eggs and J2s was observed, followed by direct parasitism of hyphae coiling around the J2s (Fig. 1b) and penetrating them (Fig. 1i), and egg colonization by the fungi (Fig. 1c). Conidia and hyphae were tightly attached to the egg surfaces (Fig. 1d, e) and appressorium-like structures were observed during penetration (Fig. 1f). Variations were observed among the different *Trichoderma* species and isolates in their attachment and parasitic capabilities (Table 1). *Trichoderma asperellum*-203 and 44 were the most prominent isolates in terms of conidial attachment and parasitism on egg masses and eggs. Many GH11 conidia were attached to nematode eggs, but few penetrated and the fungus was weakly parasitic on eggs and J2s (Table 1). *Trichoderma atroviride* exhibited less conidial adhesion to egg masses and eggs than *T. asperellum* isolates, but it was highly effective in terms of both attachment and parasitism on J2s. *Trichoderma harzianum* exhibited a very low level of attachment to the nematodes and was less effective in the parasitic process (Table 1); furthermore, its growth was not enhanced in the presence of the egg masses and germination of agglutinated conidia was inhibited by the gm.

Almost no conidial attachment was observed to gm-free eggs and J2s; nevertheless, those eggs were penetrated and colonized by the fungal hyphae (Fig. 1h) following their incubation with conidia of the different *Trichoderma* isolates. Differences were recorded in this parasitic ability among the fungal species and isolates tested. *Trichoderma asperellum*-203 was the most effective at parasitizing gm-free eggs (Table 2). The fungus parasitized mainly immature eggs while almost no penetration of mature eggs that already contained juvenile stages was observed (Table 2). *Trichoderma atroviride* exhibited the most effective parasitism of mature eggs (Table 2). Colonization by *T. asperellum*-GH11 was mainly recorded on eggshells, with low consumption of the egg contents. Conidial attachment and parasitism on gm-free J2s by the different *Trichoderma* isolates were seldom observed; *T. atroviride* exhibited the highest rate of parasitism of this stage (Table 2). Juveniles and eggs (gm-free), which were exposed to gm suspension, showed attached *T. atroviride* and *T. asperellum*-203 conidia and were parasitized by the fungi, similar to the gm-eggs and J2s.

The effect of ions on conidial attachment to the nematode life stages was tested with *T. asperellum*-203 and with *T. atroviride*, the latter exhibiting a stronger dependence on Ca^{2+} for attachment. When a Ca^{2+}-free medium was used, attachment of conidia of *T. atroviride* was markedly reduced and its parasitism on J2s dropped from 83.4% in the presence of Ca^{2+} to 12% in the absence of this ion. Addition of Mg^{2+} or Mn^{2+} instead of Ca^{2+} did not enhance conidial attachment to the nematodes; this is therefore a Ca^{2+}-dependent process.

Role of gm in J2 immobilization by *T. atroviride* and *T. asperellum* isolates

During parasitism assays with egg masses, hatched gm-J2s that were not directly parasitized by the fungus were immobilized after 24 h by *T. atroviride* and after 48 h by *T. asperellum*-203 or 44. These same fungi did not manifest this effect in assays with gm-free eggs or J2s. *Trichoderma asperellum*-GH11 did not affect J2 mobility, with or without gm. *Trichoderma harzianum* exhibited immobilization activity in the presence of gm-free eggs or J2s, but this effect was reduced in the presence of

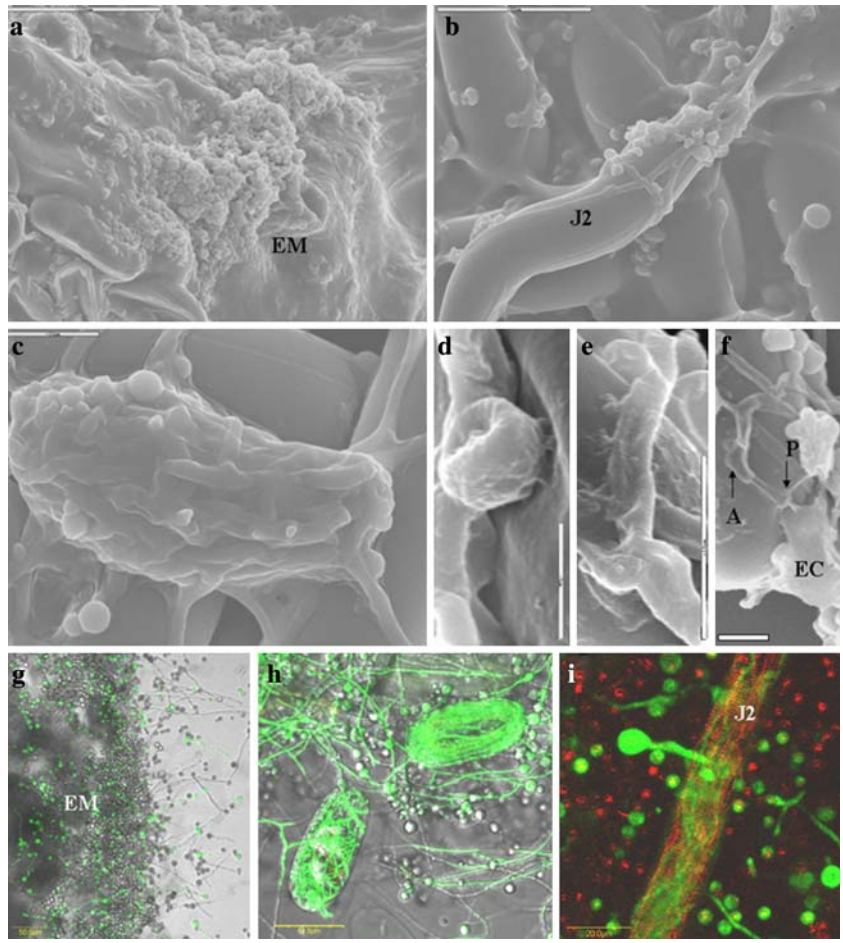

Fig. 1 Parasitism of *Trichoderma asperellum*-203 on *Meloidogyne javanica*. (a–f) Scanning electron micrographs: (**a**) Conidia attachment to an egg mass (EM), bar = 100 µm. (**b**) Fungal parasitism on egg-mass-originated juvenile (J2), bar = 50 µm. (**c**) Parasitism on egg-mass-originated egg, bar = 20 µm. (**d**) Conidium attached to egg surface, bar = 5 µm. (**e**) Hyphal attachment to egg surface, bar = 10 µm. (**f**) Appressorium-like formation on egg surface (A), penetration (P) and leakage of egg contents (EC), bar = 10 µm. (**g–i**) Parasitism visualized using a *T. asperellum*-203 construct constitutively expressing GFP: (**g**) Conidia attachment and germination on an egg mass, bar = 50 µm. (**h**) Gelatinous matrix (gm)-free egg, bar = 50 µm. (**i**) gm-originated J2, bar = 20 µm. Autofluorescence was observed under red light to visualize transverse annulations in J2s

Table 1 Parasitism of *Trichoderma* on egg-mass-originated eggs and second-stage juveniles (J2s)

	Parasitism on eggs (%)	Parasitism on J2s (%)
T. asperellum-203	95.5 a	50.5 b
T. asperellum-44	91.0 a	53.2 b
T. asperellum-GH11	21.3 c	10.5 d
T. harzianum-248	10.6 d	25.6 c
T. atroviride	75.2 b	83.4 a

Assays were performed in 96-well plates containing fungal conidia and nematodes in a diluted medium, with five replicates for each treatment. Percentages of parasitized eggs and J2s were determined after 48 h by microscopic observation. Values in columns followed by different letters are significantly different according to Tukey's test ($P = 0.05$)

Table 2 Parasitism of *Trichoderma* on gelatinous matrix (gm)-free eggs and second-stage juveniles (J2s)

	Parasitism on immature eggs (%)	Parasitism on mature eggs (%)	Parasitism on J2s (%)
T. asperellum-203	95.5 a	3.5 b	5.1 b
T. asperellum-44	82.3 b	4.7 b	6.4 b
T. asperellum-GH11	58.0 c	1.2 c	2.0 c
T. harzianum-248	52.0 c	0.5 c	1.5 c
T. atroviride	78.5 b	11.5 a	10.5 a

Assays were performed in 96-well plates containing fungal conidia and nematodes in a diluted medium, with five replicates for each treatment. Percentages of parasitized nematodes were determined after 48 h by microscopic observation. Values in columns followed by different letters are significantly different according to Tukey's test ($P = 0.05$)

gm-containing egg masses, as fungal growth was inhibited in the presence of gm (see above). J2s (gm-free) added after 48 h to the medium in the wells that had shown the aforementioned effect were also immobilized within 24 h.

Conidia agglutination by gm suspension

A quantitative assay of conidia agglutination by serially diluted gm suspensions was performed with *T. asperellum*-203 and *T. atroviride*. Protein content of the gm suspension was 65 μg ml^{-1} and the lowest protein concentration at which agglutination could be recorded, at the × 64 dilution, was about 1 μg ml^{-1} (Fig. 2a). The agglutinated conidia were spread all over the well, whereas in the control and at greater dilutions, conidial sediment was concentrated at the bottom. Conidia of both fungi exhibited this agglutination effect; however, agglutination of *T. atroviride* was highly Ca^{2+}-dependent (Fig. 2a, b), whereas that of *T. asperellum*-203 was only reduced twofold in the absence of Ca^{2+}. Mg^{2+} and Mn^{2+} ions did not cause a similar effect with these fungi.

Conidial attachment to the gm enhanced germination and hyphal growth, even when the gm served as the sole nutrient source, indicated that this material can be utilized by the fungus. Conidia that have been agglutinated in the presence of gm suspension were washed and transferred to a minimal medium; those conidia still exhibited enhanced germination and hyphal growth as compared to the control (Fig. 2c, d).

Role of carbohydrates in conidial attachment to nematodes and in agglutination by gm

Attachment of conidia of *T. asperellum*-203 to gm-J2s was inhibited by the presence of fucose or

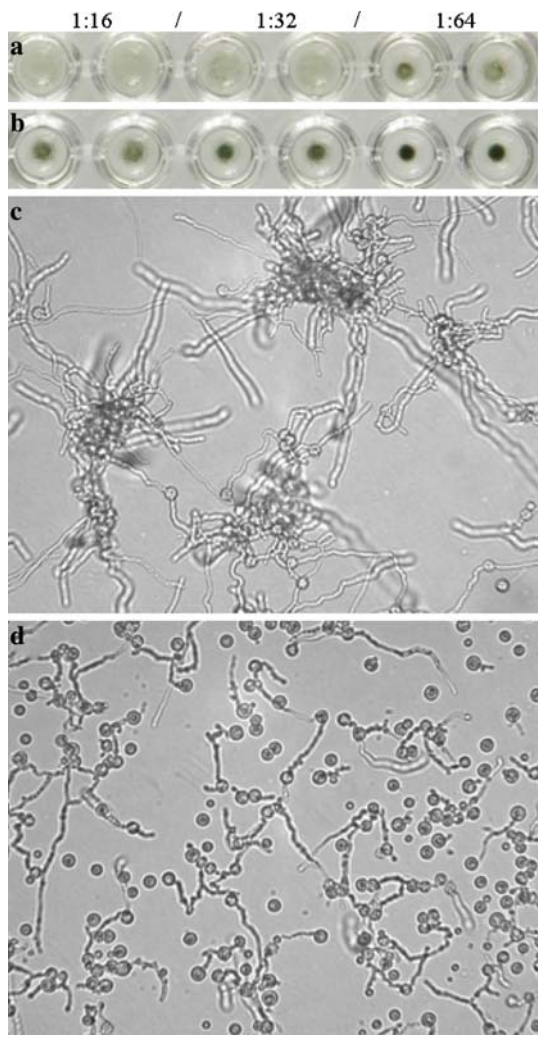

Fig. 2 Agglutination assay of *Trichoderma atroviride* conidia with (**a**) serial dilutions of *Meloidogyne javanica* gelatinous matrix (gm) suspension. (**b**) Reduced agglutination in Ca^{2+}-free buffer. (**c**) Germination of gm-agglutinated and washed *Trichoderma atroviride* conidia in a diluted medium after 24 h. (**d**) Control, untreated conidia

α-methyl-manoside in the assay media, or following pre-incubation of conidia with those carbohydrates. In the gm agglutination assay, pre-treatment of conidia with fucose, α-methyl-manoside, glucose or galactose slightly reduced agglutination (twofold), whereas *N*-acetyl-glucosamine and *N*-acetyl-galactosamine had no effect on agglutination.

Egg masses, gm-eggs and -J2s were treated with sodium-periodate to oxidize surface carbohydrates; *T. asperellum*-203 conidia were also treated. Exposure of the different life stages to periodate completely inhibited the attachment of non-treated conidia, whereas periodate-pre-treated conidia adhered normally to the non-treated nematodes, although their germination was reduced compared to non-treated controls. Citrate buffer had no effect on conidial or nematode behaviour.

Biomimetic system: binding of gm to nylon fibers

A biomimetic system based on nylon fibers was used to demonstrate the role of nematode gm in conidial attachment and induction of parasitic growth patterns. *Trichoderma asperellum*-203 (Fig. 3a) and *T. atroviride* conidial became attached to gm-treated nylon fibers. Hyphae were tightly attached to the gm-coated fibers; a coiling growth pattern, branching, enlargement of the hyphal tips and appressoria-like structures were observed (Fig. 3b–d). Control treatments with BSA-coated fibers did not result in conidia attachment (Fig. 3e). Fiber-attached conidia exhibited enhanced germination relative to the unattached conidia in the medium, and to the controls. *Trichoderma asperellum*-203 conidia exhibited higher attachment ability than those of *Trichoderma atroviride* (12.5 ± 2.2 vs. 5.7 ± 1.2 conidia 100 μm^{-1} fiber, respectively).

Attachment of *T. asperellum*-203 conidia to fucose-treated gm fibers and the subsequent parasitic growth patterns were reduced, although initial adhesion (observed after 1 h) was normal. The average number of conidia attached to the gm-treated fibers was 12.5 ± 2.2 per 100 μm of fiber, whereas when covered with fucose-treated gm, attachment was only 4.3 ± 1.5 per 100 μm of fiber. The biomimetic system showed that gm-coated fibers specifically triggered conidial attachment and fungal parasitic behaviour patterns, which could be inhibited by fucose.

Fungus-nematode interactions *in planta*

Observations made during root penetration by the nematode on tomato roots pre-colonized with constitutively expressing GFP-*T. asperellum*-203 showed that the fungus colonizing the roots interacts with the penetrating J2s and colonizes their penetration sites

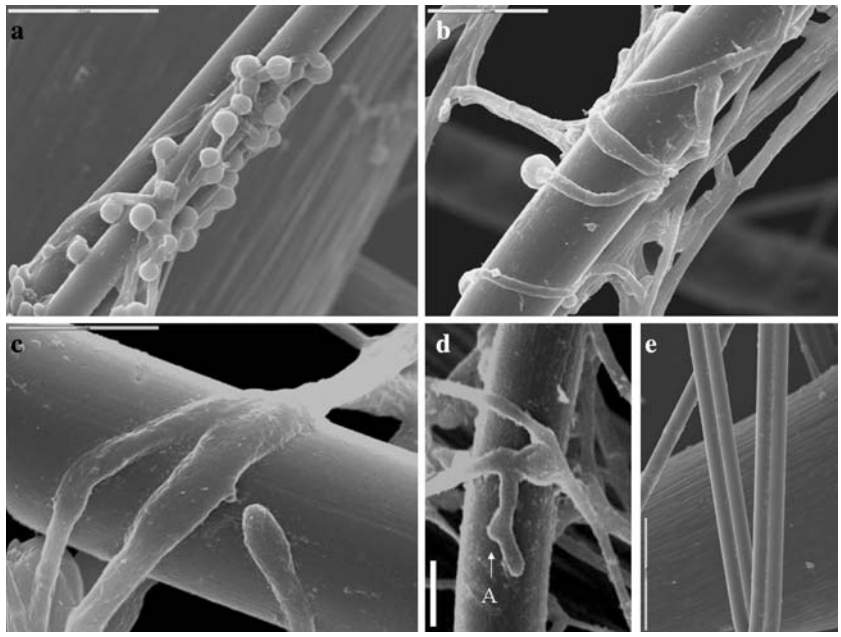

Fig. 3 Scanning electron micrographs of nylon fibers coated with gelatinous matrix (gm). (**a**) Conidia attachment, bar = 50 μm. (**b**) Fungal parasitic-like behaviour: coiling and branching, bar = 20 μm. (**c**) Tight adhesion of hyphae, branching and tip enlargement, bar = 10 μm. (**d**) Appressorium-like (A) structure, bar = 10 μm. (**e**) BSA controls showed almost no conidium attachment, bar = 100 μm. Fibers were incubated with (**a**,**b**) *Trichoderma asperellum*-203 or (**c**,**d**) *T. atroviride* conidia

(Fig. 4a). These *Trichoderma*- treated roots developed significantly less initial galls than control roots without the fungus (11.5 ± 2.4 and 5.3 ± 2.2 respectively) 3 days after nematode inoculation in this sterile system.

Biocontrol activity in soil

Trichoderma isolates exhibited significant nematode biocontrol activity in growth-chamber pot experiments. All *Trichoderma* treatments improved top fresh weights, and reduced both the galling indices and the number of infective J2 that hatched from the egg masses on roots (Table 3). The peat-bran alone caused some improvement in plant growth, but it did not reduce galling index, though it slightly reduced J2s hatching (Table 3).

Parasitism *in planta* on roots from the pot experiments was demonstrated on *T. asperellum*-203 treated roots where females and egg masses were dissected from roots. Females (61%) were found to be infected by a fungus (Fig. 4b) and colonized eggs were found within 65% of the egg masses (Fig. 4c). Such fungal parasitism was not observed in the control treatments without *Trichoderma*.

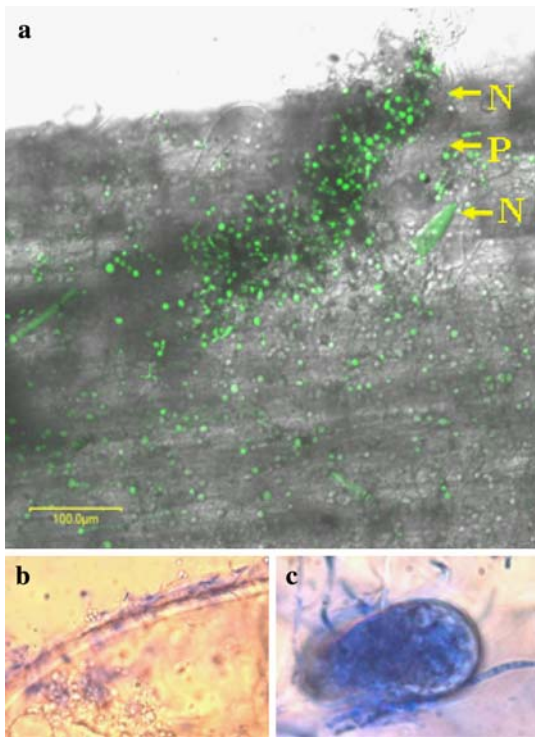

Fig. 4 Interactions of *Trichoderma asperellum*-203 with *Meloidogyne javanica* developing stages *in planta*. (a) Colonization of tomato roots by GFP-expressing construct and interaction with second-stage juveniles (J2s) during root penetration in sterile soil; nematode (N) penetration site (P) colonized by the fungus. A penetrating nematode showed green autofluorescence, probably indicating loss of viability. Bar = 100 µm. (**b**–**c**) Parasitism of *T. asperellum*-203 on mature nematode life stages dissected from tomato roots grown in *Trichoderma*-treated soil: (**b**) Female infected by the fungus. (**c**) Egg within the egg mass colonized by the fungus. The fungus was stained with aniline blue

Discussion

This study was aimed at elucidating the parasitic capabilities of *Trichoderma* isolates on the RKN, *M. javanica* and their biocontrol activities against the nematode. Parasitism is probably an important mode of action and one of the initial steps of this process is attachment. The nematode's gm enabled fungal attachment and enhanced parasitic capabilities of the isolates (except *T. harzianum*), which could also utilize gm as a nutrient source.

The gm has also been found to trigger proteolytic and chitinolytic enzyme production by the fungus (Sharon et al. unpubl.). This combination of enzymes is required to disrupt the eggshell (Tikhonov et al. 2002; Khan et al. 2004), although chitinolytic capacity is probably the most important activity on the eggshells (Morton et al. 2004). *Trichoderma asperellum* GH-11 exhibited lower parasitic capabilities that might be related to insufficient proteolytic activity of this isolate, while *T. atroviride* presented the greatest efficiency for parasitism of J2s, probably because of its high proteolytic activities (Sharon et al. unpubl.). Production of proteinase Prb1 in this isolate has been studied and its involvement in fungal parasitism has been shown (Flores et al. 1997). A transgenic *T. atroviride* line (P2) containing multiple copies of the *prb1* gene also exhibited improved biocontrol activity of *M. javanica* in soil experiments and in parasitism assays on agar in vitro (Sharon et al. 2001). The gm also triggered an immobilization effect on J2s produced by *T. atroviride* and *T. asperellum* isolates 203 and 44. This effect might be the result of enzymes and metabolites, such as peptaibols, the activities of which may act synergistically. Parallel formation and synergism of hydrolytic

Table 3 Biocontrol activity of *Trichoderma* species and isolates against *Meloidogyne javanica* on tomato plants

Treatments	Top fresh weight (g per plant)	Galling index (1–5 scale)	Juveniles (J2s) per g root
T. asperellum-203	37.3 a	0.3 b	103 c
T. asperellum-44	38.2 a	0.7 b	540 b
T. asperellum-GH11	38.5 a	1.0 b	565 b
T. harzianum-248	35.1 a	1.2 b	667 b
T. atroviride	43.2 a	1.0 b	770 b
Control nematodes	15.4 b	4.1 a	11,000 a
Control peat-bran	20.5 b	3.7 a	9,000 a

Experiments were conducted in 1.5 l pots with peat-bran preparations (1% w/w) of various *Trichoderma* isolates. Fungi were mixed with nematode-infested soil 2 weeks before tomato seedlings were planted. Non-treated, nematode-infested soil and peat-bran-amended soil (1% w/w) served as controls. Each treatment included eight replicates. Six weeks after planting, the plants were uprooted and examined

Values in columns followed by different letters are significantly different at $P = 0.05$ according to Tukey's HSD test

enzymes and peptaibol antibiotic action against phytopathogenic fungi has been reported in *Trichoderma* (Schirmböck et al. 1994; Kubicek et al. 2001).

Conidial attachment and parasitic processes were microscopically monitored in vitro. Hyphal coiling, branching, enlargement of hyphal tips and appressoria-like structures were observed during parasitism on nematodes, resembling the mycoparasitic behaviour of *Trichoderma* (Chet et al. 1997). Nematode egg surfaces are infected by some fungal endoparasites, such as *Pochonia chlamydosporia* (*Verticillium chlamydosporium*) and *Paecilomyces lilacinus*, by producing appressoria laterally or at the tip of the hyphae growing across the eggs (Kerry and Hominick 2001). The biomimetic system successfully expressed the specific triggering of fungal attachment and parasitic growth patterns by the gm, similar to the parasitism on the nematodes. It was also similar to those induced by lectins derived from host fungi (Inbar and Chet 1994). Coiling on nylon fibers has been induced by several commercial lectins, such as concanavalin-A (Con-A), wheat-germ agglutinin (WGA) and *Ulex europaeus*-I (gorse, UEA-I) (Rocha-Ramirez et al. 2002).

Hyphae of *T. atroviride*, which was the most effective parasite of the J2s, showed a higher tendency to coil around the J2s than those of *T. asperellum*-203. Similar results with respect to the coiling process have been obtained in fungal–fungal biomimetic interactions using nylon fibers, especially after induction with a G-protein activator (Omero et al. 1999). The signal-transduction pathways downstream of the recognition event have recently been intensively investigated, with a focus on the role of G-protein α-subunit genes (Zeilinger et al. 2005). Further investigations may determine whether similar pathways are involved in gm induction of fungal parasitic behaviour.

Fucose inhibited conidial attachment to J2s, conidial agglutination by gm suspension and their attachment to nylon fibers; attachment was also inhibited after periodate treatment of nematodes. It is suggested that carbohydrate lectin-like interactions might be involved in these processes; such interactions are sometimes Ca^{2+}-dependent. This was the case for binding of red blood cells to nematode surfaces, where the presence of C-type fucose-, mannose- and glucose-binding proteins (carbohydrate-recognition domains, CRDs) on the J2s was suggested (Spiegel et al. 1995). The nematode surface coat also contains carbohydrate residues, including fucose and mannose (Spiegel and McClure 1995). Information about the composition of the gm is scarce. Its amino-acid and amino-sugar contents have been analyzed and some glycoproteins have been characterized (Sharon and Spiegel 1993). A gm suspension was specifically labelled by several lectins, indicating the presence of carbohydrates such as fucose and *N*-acetyl-glucosamine (Sharon and Spiegel 1993). The conidial surface probably contains CRDs and carbohydrates: Elad et al. (1983) reported that attachment of *T. asperellum*-203 to *Rhizoctonia solani* is inhibited by galactose and fucose, and is Ca^{2+}- and Mn^{2+}-dependent. Following this work, Barak et al. (1986) isolated a fucose-binding agglutinin from the host fungus. We suggest that during J2s

hatch from egg mass, gm, which contains carbohydrates such as fucose, binds to the J2s surface coat and this can alter their binding affinity to the fungal conidia that contain fucose-binding domains. As a result, gm-J2s are efficiently attached and parasitized by the fungus.

The results suggest that *M. javanica* gm plays a key role in the process of *Trichoderma* conidial attachment to the nematode and in the ensuing parasitism. The gm is usually considered a defensive envelope that protects the eggs against microorganisms and enables the egg mass to survive in the soil (Orion et al. 2001). Bacteria that were agglutinated by the gm could not reproduce in its presence, whereas others, which were not agglutinated, utilize the gm as a nutrition source and reproduce (Sharon et al. 1993). Thus, the ability of some *Trichoderma* species to be agglutinated by the gm and grow on it is unique, and partially accounts for their ability to attack RKNs; in contrast, the *T. harzianum* isolate was inhibited by the gm and was therefore not an effective parasite on the nematodes.

The potential ability to parasitize nematode life stages *in planta* was demonstrated with *T. asperellum*-203, which interacted with penetrating J2 in a sterile soil system, and with females and egg masses on roots in soil, thereby interfering with the reproduction process. The potential parasitic capability of this isolate on the different nematode life stages may partially account for its high efficacy at reducing root galling and viable egg production in soil experiments. The high affinity of this isolate as a root-surface colonizer (Yedidia et al. 1999) probably enhances these parasitic fungus-nematode interactions on the root surface. Kok et al. (2001) reported that the egg masses of *Meloidogyne* species from soils contained a bacterial community significantly greater than that of the rhizosphere. They suggested that the egg masses microflora may be an important factor in determining the success of nematode biocontrol agents. Interestingly, a strain of *Trichoderma* that strongly reacted against the biocontrol agent *V. chlamydosporium* was found among *M. fallax* egg masses microflora.

This work demonstrated the biocontrol activity of different *Trichoderma* isolates. Differences were observed among the isolates in their in vitro attachment and parasitic capabilities. Parasitism is one of the possible modes of action of *Trichoderma* against the nematodes; however, it is not always correlated with the biocontrol activity recorded among the different *Trichoderma* species and isolates, suggesting the involvement of other additional mechanisms. Understanding fungus-nematode interactions and the mechanisms involved in the biocontrol process for various *Trichoderma* species and isolates might contribute to the development of improved biocontrol agents and their use with optimal implementation methods.

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